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Hypotonicity-induced Reduction of Aquaporin-2 Transcription in mpkCCD Cells Is Independent of the Tonicity Responsive Element, Vasopressin, and cAMP*

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The syndrome of inappropriate antidiuretic hormone secretion is characterized by excessive water uptake and hyponatremia. The extent of hyponatremia, however, is less than anticipated, which is ascribed to a defense mechanism, the vasopressin-escape, and is suggested to involve a tonicity-determined down-regulation of the water channel aquaporin-2 (AQP2). The underlying mechanism, however, is poorly understood. To study this, we used the mouse cortical collecting duct (mpkCCD) cell line. MpkCCD cells, transfected with an AQP2-promoter luciferase construct showed a reduced and increased AQP2 abundance and transcription following culture in hypotonic and hypertonic medium, respectively. This depended on tonicity rather than osmolality and occurred independently of the vasopressin analog dDAVP, cAMP levels, or protein kinase A activity. Although prostaglandins and nitric oxide reduced AQP2 abundance, inhibition of their synthesis did not influence tonicity-induced AQP2 transcription. Also, cells in which the cAMP or tonicity-responsive element (CRE/TonE) in the AQP2-promoter were mutated showed a similar response to hypotonicity. Instead, the tonicity-responsive elements were pin-pointed to nucleotides −283 to −252 and −157 to −126 bp. In conclusion, our data indicate that hypotonicity reduces AQP2 abundance and transcription, which occurs independently of vasopressin, cAMP, and the known TonE and CRE in the AQP2-promoter. Increased prostaglandin and nitric oxide, as found *in vivo*, may contribute to reduced AQP2 in vasopressin-escape, but do not mediate the effect of hypotonicity on AQP2 transcription. Our data suggest that two novel segments (−283 to −252 and −157 to −126 bp) in the AQP2-promoter mediate the hypotonicity-induced AQP2 down-regulation during vasopressin-escape.

Renal water reabsorption is regulated by the hormone arginine vasopressin (AVP).[2] AVP binding to its V2 receptor in renal principal cells induces a cAMP cascade leading to increased translocation of the water channel aquaporin-2 (AQP2) to the apical membrane, resulting in water reabsorption from the pro-urine (1). In addition to this short-term effect, cAMP increases AQP2 transcription through phosphorylation of the transcription factor CREB (cAMP responsive element-binding protein), which then binds to a cAMP responsive element (CRE) at −210 in the AQP2 promoter (2, 3). The increase in transcription is a more long-term effect, requiring hours to take effect.

AVP synthesis and release are regulated by alterations in plasma osmolality as well as non-osmotic, baroreceptor-mediated pathways (4). Osmolality not only affects plasma AVP, but also appears to have direct effects on urine concentrating ability and AQP2 expression. In the syndrome of inappropriate antidiuretic hormone secretion (SIADH), for example, levels of AVP are inappropriately high relative to plasma osmolality, resulting in free-water retention and hypotonicity. Under these circumstances, however, the free-water excretion is considerably higher than would be expected from the vasopressin concentrations (5). This phenomenon, called the vasopressin-escape, indicates that there are mechanisms counteracting vasopressin action. In a rat animal model, it has been shown that the onset of this escape coincides with a decrease in the AQP2 expression in the renal collecting duct (6). Hypotonicity and/or volume expansion have been proposed to mediate this AVP-independent direct regulation of AQP2 expression (7).

Alternatively, an AVP-independent increase of AQP2 expression has been suggested to occur with hypertonicity. Valtin and Edwards (8) already reported that water deprivation of vasopressin-deficient Brattleboro rats leads to hypertonicity, increased papillary interstitial osmolality, and concentration of the urine. Similarly, Li *et al.* (9) found that hypertonicity due to hyperglycemia or increased NaCl levels increases AQP2 expression in Brattleboro rats.

At present, it is still largely unknown how the AVP-independent expression of AQP2 is regulated at the molecular level. In mouse-collecting duct principal cells ($mpkCCD_{c14}$), Hasler *et al.* (10) showed that hypertonicity decreases the expression of AQP2 in the short term, but increases its expression in the long term. Here, hypertonicity did not affect the stability of AQP2 mRNA or protein, indicating that hypertonicity increases AQP2 abundance by increasing its transcription.

One of the proteins suggested to be involved in the hypertonicity response is the tonicity-responsive enhancer-binding protein (TonEBP). This protein is up-regulated with hyperto-

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[2] The abbreviations used are: AVP, arginine vasopressin; AQP2, aquaporin-2; mpkCCD, mouse cortical collecting duct; TonE, tonicity-responsive element; TonEBP, tonicity-responsive enhancer-binding protein; dDAVP, 1-*deamino*-8-D-arginine vasopressin.

TABLE 1
Primers used to generate AQP2 promoter reporter constructs

Primer	Forward primer (5′-3′)	Reverse primer (5′-3′)
Deletion 1 (−408 to −377 bp)	ggggtaccgtttggggtaaggcattg	cgtcttccatggtggctttacc
Deletion 2 (−377 to −346 bp)	ccacatttcctcacaagccttttagtc	gactaaaaggcttgtgaggaaatgtgg
Deletion 3 (−346 to −315 bp)	cttccttggccacagcctcctctgc	cagaggaggctgtggccaaggaagg
Deletion 4 (−315 to −283 bp)	aggtcactggactcattgtgggggctg	agcccccacaatgagtccagtgaccttc
Deletion 5 (−283 to −252 bp)	tggtgctggtgggctccatggggtaac	taccccatggagcccaccagcaccag
Deletion 6 (−252 to −220 bp)	cagccctgaggcaaaacagagacgtc	gacgtctctgttttgcctcagggctgc
Deletion 7 (−220 to −189 bp)	ctgaggaaaaaacgaggaattaatgaggag	ctcctcattaattcctcgtttttttcctcag
Deletion 8 (−189 to −157 bp)	cttatctggagtccgctaagatggggtg	caccccatcttagcggactccagataag
Deletion 9 (−157 to −126 bp)	gtcagctgtgaaacaggagcagggatg	catccctgctcctgtttcacagctgac
Deletion 10 (−126 to −95 bp)	ttcgggtgggggggagcacagggttg	aaccctgtgctccccccacccgaag
Deletion 11 (−95 to −64 bp)	aagtccgccatgccaccccacgtgc	cacgtggggtggcatggcggacttg
Deletion 12 (−64 to −34 bp)	caggaacgcctcctggccctataagtg	cacttatagggccaggaggcgttcctg
Mutation CRE	cgaggaaaacagagtggtcaatccttatctggagtcc	ggactccagataaggattgaccactctgttttcctcg
Mutation TonE	ccaagacctttttgccttttaaatttgtcccaggccagccag	ctggctggcctgggacaaatttaaaaggcaaaaggtcttgg

nicity in cultured cells and in the kidney medulla (11, 12) and TonEBP knockdown or inhibition, but also mutation of its tonicity-responsive element (TonE) in the AQP2 promoter, reduced AQP2 expression in mpkCCD cells (13, 14). Moreover, in TonEBP$^{-/-}$ mice and mice transgenic for dominant-negative TonEBP, AQP2 expression was decreased (15, 16). However, the role of TonEBP and its TonE element in AQP2 expression regulation is still controversial. In Madin Darby Canine Kidney (MDCK) cells, for example, Kasono *et al.* observed a hypertonicity-induced increase in AQP2 transcription only when the AQP2 promoter was −6.1 kb or longer and therefore did not involve TonE, which localizes at 489 bases upstream of the AQP2 transcription start site (7). Moreover, the severe atrophy of the renal medulla in the TonEBP$^{-/-}$ and dominant-negative TonEBP-overexpressing mice complicates the interpretation of its direct involvement in AQP2 regulation.

In addition to TonE, other promoter elements have been suggested to be involved in hypertonicity-induced AQP2 expression, as Li *et al.* (14) reported that NFATc binding sites downstream of TonE are also important for hypertonicity-induced AQP2 expression in mpkCCD cells, because following mutation of all five sites, hypertonicity-induced AQP2 expression was lost to a large extent.

Thus far, the effects of hypotonicity on AQP2 expression and the underlying mechanisms have not been studied in great detail. Thus, it is unclear whether the regulatory changes during hypotonicity mirror the regulation under hypertonic conditions and involve changes in TonEBP or in the classical AVP-cAMP signaling cascade elements. To decipher this, we tested and used the mpkCCD cell line, which shows dDAVP-induced expression of endogenous AQP2 (17).

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse mpkCCD$_{cl4}$ cells (18) were grown in a modified defined medium (DMEM:Ham's F12 1:1 v/v; 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose, 2% fetal calf serum, and 20 mM HEPES (pH 7.4)), resulting in a medium of 355 mOsm. Cells were seeded at a density of 1.5×10^5 cells/cm^2 on semi-permeable filters (Transwell®, 0.4-μm pore size, Corning Costar, Cambridge, MA) and cultured for 8 days. Unless stated otherwise, the cells were treated for the last 96 h with 1 nM 1-*deamino*-8-D-arginine vasopressin (dDAVP) at the basolateral side, to induce AQP2 expression. When used H89 (10 μM, Calbiochem, San Diego, CA) was added to both the apical and basolateral side. Tonicity was changed by adding solute to NaCl-free defined medium (made identical to defined medium used above). Tonicity was changed at both sides of the cells. Osmolality was measured using a Knauer K-7400 semi-micro osmometer (Berlin, Germany).

Constructs—To generate an AQP2 promoter-luciferase reporter construct (pGL3-AQP2–3.0-luc), we first added the neomycin selection marker to pGL3-basic (pGL3-neo). For this, the neomycin cDNA preceded by an SV40 promoter was cut from pcDNA 3.1 (Invitrogen) using XmnI and SalI, and ligated into a blunted BamHI and SalI site of pGL3-basic (Promega). Next, a 3.3-kb fragment, containing the mouse AQP2 promoter (−2970 to +60 upstream of the transcription start site) was obtained by PCR using the primers GAAGATCTGGTCTGGGCCACGGGGGTGGCTCTTCC and GGTGCACCGAGCCTCCTCCTCAGCC on mouse genomic DNA. Following digestion with BglII, which cuts the primer (restriction site underlined) and at −2970 in the AQP2 promoter, the product was ligated into the BglII site upstream of the luciferase cDNA in pGL3-neo.

For pGL3-AQP2–0.4-luc (containing −408 to +60 of the AQP2 promoter), pGL3-AQP2–3.0-luc was cut with SacII and KpnI, blunted, and religated. Constructs lacking 31-bp segments of the −0.4 kb promoter were made by 3 point PCR using different primers (Table 1) with pGL3-AQP2–0.4-luc as a template. Mutations in CRE were introduced in pGL3-AQP2–0.4-luc using site-directed mutagenesis (Stratagene) with mutagenesis primers listed in Table 1. This mutation has been shown to inactivate the AQP2 promoter (2). Mutations in TonE, reported to reduce hypertonicity-induced AQP2 transcription (13), were introduced in pGL3-AQP2–3.0-luc using primers as shown in Table 1. To generate pGL3-CRE(21)-luc, a DNA fragment containing 21 CRE-elements was cut from pCRE121–3 (19) with SalI and XhoI and ligated into the XhoI site of pGL3-neo. Proper orientation and sequences were confirmed by sequence analysis.

MpkCCD cells were stably transfected using the calcium phosphate precipitation technique as described (20). Transfected colonies were selected with G418 (0.25 mg/ml) and pooled to level out differences between individual colonies.

Luciferase Assay—Luciferase activity was measured using the Luciferase Assay System (Promega) following the manufactur-

er's instructions. Luminescence was measured for 10 s using an EG&G Berthold Lumat LB9507 luminometer (Bad Wildbad, Germany). To verify that equal amounts of protein per sample were used for the luciferase assay, protein concentration was determined using the Bio-Rad protein assay as described (21). Light absorbance at 595 nm was measured using a helios omega spectrophotometer (Thermo Scientific, Rockford, IL).

Immunoblotting—mpkCCD$_{cl4}$ cells from 1.13 cm^2 filter were lysed in 200 μl of Laemmli/DTT buffer, sonified, and incubated for 30 min. at 37 °C. PAGE, blotting, and blocking of the PVDF membranes were done as described (22). Membranes were incubated overnight with 1:3000-diluted affinity-purified rabbit R7 AQP2 (23), 1:2000 rabbit CREB (Sigma) or 1:2000 rabbit CREB-pS133 antibodies (Sigma) in Tris-buffered saline Tween-20 (TBS-T) supplemented with 1% w/v nonfat dried milk. Blots were incubated for 1 h with 1:5000-diluted goat anti-rabbit IgGs (Sigma) as secondary antibody coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce). Densitrometric analyses were performed using Bio-Rad quantification equipment (Bio-Rad 690c densitometer, Chemidoc XRS) and software (QuantityOne). Equal loading of the samples was confirmed by staining of the blots with Coomassie Blue G250 (Serva, Heidelberg, Germany).

cAMP Assay—MpkCCD cells were seeded on filters for 8 days, either with or without 1 nM dDAVP for the last 4 days. The last 24 h, cells were grown in hypotonic or isotonic medium. The phosphodiesterase inhibitor 3-isobutyl 1 methylxanthine (IBMX) was added to both the apical and basolateral medium to a final concentration of 500 μM 30 min before harvesting. In the cells grown without dDAVP, dDAVP was added to the basolateral side 15 min before harvesting. cAMP was measured using the cAMP-Glo assay (Promega) according to the manufacturer's instructions.

Statistical Analyses—Student's *t* test was applied to compare two groups with Gaussian distribution. Levene's test was used to compare variances. *p* values below 0.05 were considered significant. For multiple comparisons, Bonferroni correction was applied.

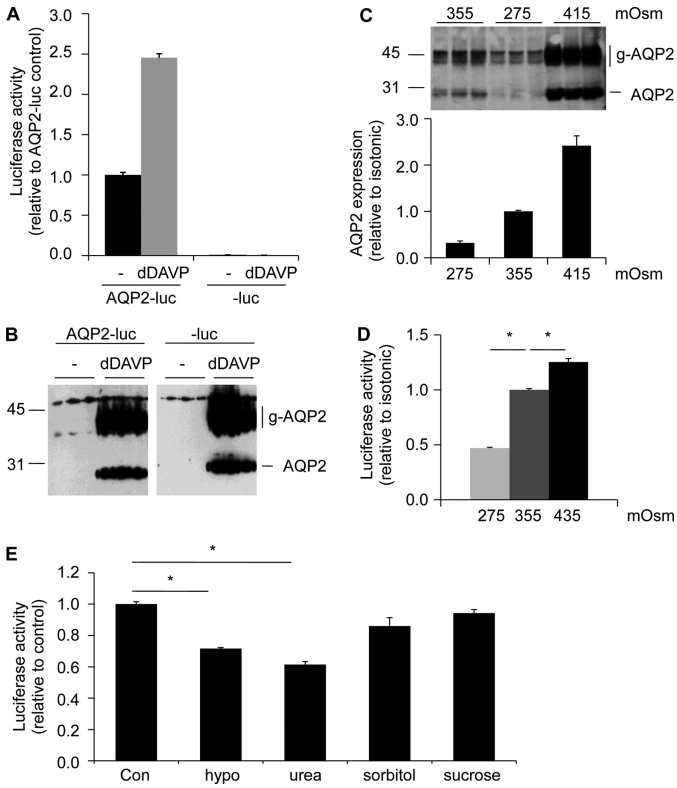

FIGURE 1. **mpkCCD cells as a model for tonicity-induced AQP2 expression.** *A* and *B*, mpkCCD-AQP2-luc and mpkCCD-luc cells were grown to confluence, the last 4 days with or without dDAVP. Cells were lysed and analyzed for luciferase activity (*A*) or for AQP2 expression (*B*). Immunoblotting the lysates for AQP2 revealed non-glycosylated (AQP2; 29 kDa) and complex-glycosylated (g-AQP2; 40–45 kDa) AQP2 in lanes of mpkCCD cells treated with dDAVP. Molecular mass (in kDa) is indicated on the *left*. *C* and *D*, MpkCCD-AQP2-luc cells were grown to confluence and treated with 1 nM dDAVP for 4 days. Over the last 24 h, cells were exposed to different osmolalities (indicated) using NaCl. Cells were lysed and analyzed for AQP2 expression (*C*) and luciferase activity (*D*). *E*, mpkCCD-AQP2-luc cells were grown as above. During the last 24 h, cells were grown in hypo-osmotic medium, containing 80 mM NaCl (hypo 275 mOsm), or medium with a osmolality of 355 mOsm, by adding NaCl, (*Con*) urea, sorbitol or sucrose (indicated). Cells were lysed, and luciferase activity was determined. In all experiments above, the mean values of luciferase activity per condition (±S.E.) were determined from three independent filters per condition. Significant differences ($p < 0.05$) are indicated by an *asterisk*.

RESULTS

mpkCCD Cells as a Model for Tonicity-induced AQP2 Expression—Hypertonicity has been shown to affect AQP2 transcription (10). To develop an easy readout cellular system for AQP2 transcription and to test whether mpkCCD cells are a proper cell model for these analyses, we generated pooled colonies of mpkCCD cells stably transfected with the pGl3-AQP2–3.0-luc construct (mpkCCD-AQP2-luc), or a construct lacking the AQP2 promoter (mpkCCD-luc). To first test whether the promoter conferred vasopressin sensitivity, the mpkCCD-AQP2-luc and mpkCCD-luc cells were grown on semi-permeable filters for 8 days of which the last 4 days with or without 1 nM dDAVP (17). Subsequent analysis revealed a 2.5-fold higher luciferase activity in mpkCCD-AQP2-luc cells with dDAVP than without dDAVP, while luciferase activity was hardly observed in mpkCCD-luc cells (Fig. 1*A*). As endogenous AQP2 abundance was increased in both pooled colonies treated with dDAVP (Fig. 1*B*), it was concluded that the dDAVP-induced luciferase activity in mpkCCD-AQP2-luc cells is due to AQP2 promoter-specific transcription. To test whether changes in osmolality affect AQP2 expression in mpkCCD-AQP2-luc cells, cells were grown for 8 days in isotonic medium (120 mM NaCl; 355 mOsm/kg H_2O). dDAVP was added during the last 4 days. In the last 24 h, the cells were exposed to media made hypotonic or hypertonic using different concentrations of NaCl. Following lysis, immunoblotting revealed that a decreased osmolality coincided with reduced AQP2 abundance and *vice versa* (Fig. 1*C*). This was reflected in transcription from the 3-kb AQP2 promoter luciferase reporter construct, because luciferase activity increased with hyperosmolality and decreased with hypo-osmolality (Fig. 1*D*).

Changes in the concentration of NaCl affect both the osmolality and tonicity. To determine whether a change in osmolality or in tonicity regulates AQP2 transcription in mpkCCD cells, cells were grown and treated with dDAVP as described above. Over the last 24 h, cells were incubated in hypotonic medium

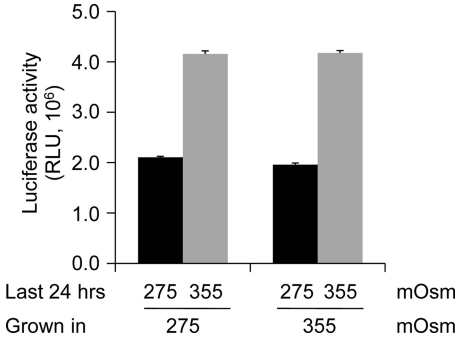

FIGURE 2. **The final tonicity, and not the change in tonicity, determines the effect on AQP2 expression.** mpkCCD-AQP2-luc cells were grown in isotonic (355 mOsm) or hypotonic (275 mOsm) medium for 8 days. In both conditions, cells were incubated in hypotonic or isotonic medium during the last 24 h. *RLU*, relative light units. Mean values of luciferase activity (±S.E.) were determined from three independent filters per condition.

(275 mOsm) or in a medium of 355 mOsm of which 80 mOsm was made up by different solutes. Subsequent luciferase activity measurements revealed that cell impermeant solutes such as sorbitol and sucrose mimicked the effect of NaCl, whereas a cell permeant solute such as urea had no effect (Fig. 1*E*). Thus, these data indicate that changes in tonicity, rather than osmolality, affect AQP2 transcription in both directions and that the 3.0 kb AQP2 promoter luciferase construct is a useful read-out system. To simulate the *in vivo* situation of hyponatremia, NaCl was used to change tonicity in all further experiments.

Does the Final or Change in Tonicity Determine AQP2 Expression?—At present, it is unclear whether the change in tonicity or the tonicity itself determines AQP2 expression. To test this, dDAVP-treated mpkCCD-AQP2–3.0-luc cells were grown in medium of 355 or 275 mOsm for 8 days. In the last 24 h, medium was either unchanged or replaced by a medium of the other osmolality. Analysis of the luciferase activity revealed that under both conditions, AQP2 transcription was 2-fold higher in cells grown for the last day in 355 *versus* 275 mOsm medium (Fig. 2). These experiments clearly indicate that the final, and not the change in tonicity, determines AQP2 expression.

Role of Nitric Oxide and Prostaglandins—Murase *et al.* (24) reported that endogenous nitric oxide (NO) synthesis was significantly increased in vasopressin-escape rats and that inhibition of NO synthesis with L-NAME decreased urine volume and partially restored AQP2 expression. In addition, the excretion of prostaglandin E_2, which is known to reduce AVP-stimulated water reabsorption in the collecting duct (25, 26), is increased in vasopressin-escape and prevention of this increase with the prostaglandin synthesis blocker indomethacin resulted in a delay in the onset of escape (27). To test whether inhibition of NO synthesis and prostaglandins directly affect tonicity-induced changes in AQP2 expression, mpkCCD-AQP2–3.0-luc cells were grown and treated with dDAVP as above, but for the last 24 h changed to medium with different osmolalities in the presence or absence of 10 μM indomethacin or 1 mM L-NAME. Immunoblot analysis of AQP2 levels revealed that indomethacin and L-NAME treatment resulted in increased AQP2 protein expression levels (Fig. 3, *A* and *B*). This increase with indomethacin and L-NAME was observed for

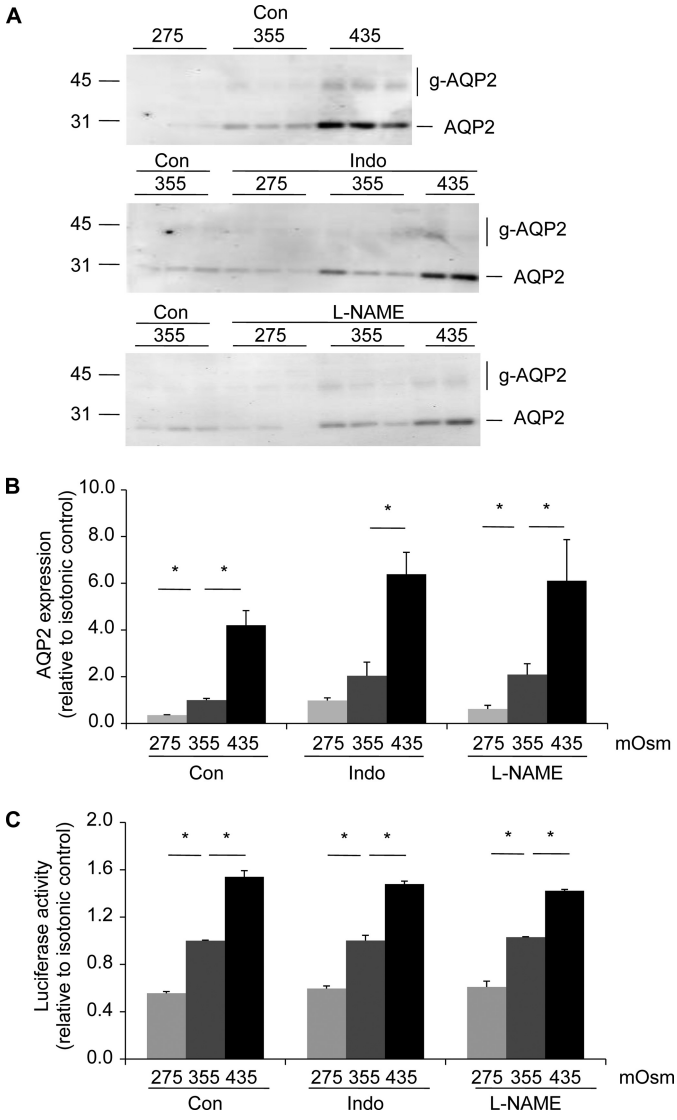

FIGURE 3. **Role of nitric oxide and prostaglandins in tonicity-regulated AQP2 expression.** *A*, mpkCCD-AQP2-luc cells were grown to confluence, the last 4 days with dDAVP. Over the last 24 h, cells were exposed to different osmolalities (indicated) in the absence or presence of indomethacin or L-NAME, blockers for prostaglandin and nitric oxide synthesis, respectively. Cells were lysed and analyzed for AQP2 abundance by immunoblotting. The molecular mass (in kDa) is indicated on the *left*. *B*, relative abundances of AQP2 derived from: *A*, normalized for AQP2 abundance of control cells grown under isotonic conditions. *C*, of samples as described above, luciferase activity was determined. Significant differences ($p < 0.05$) are indicated by an *asterisk*.

every tonicity tested. However, comparison of the AQP2 expression levels revealed no differences in the tonicity response between control-, indomethacin-, or L-NAME-treated cells. Moreover, AQP2-promoter-driven transcription of luciferase was not changed with L-NAME or indomethacin at any of the tonicities tested (Fig. 3*C*). These data indicate that, although NO and prostaglandins decrease AQP2 abundance, they do not affect tonicity-induced changes in AQP2 expression.

dDAVP Dependence of Tonicity-induced AQP2 Transcription—Tonicity can change AQP2 abundance independent of AVP *in vivo* (7, 9). To test whether an AVP-independent effect of tonicity on AQP2 expression is also observed in our cells, mpkCCD-AQP2–3.0-luc cells were again treated with dif-

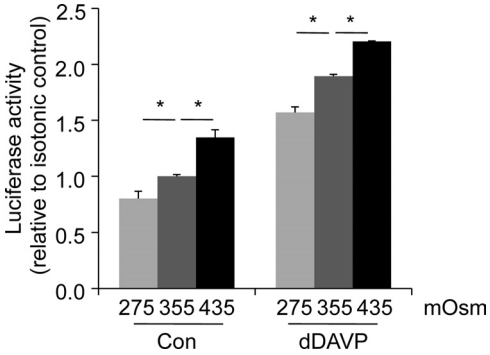

FIGURE 4. **Role of dDAVP in tonicity-induced AQP2 transcription.** mpkCCD-AQP2-luc cells were grown as above, the last 4 days with or without (*Con*) dDAVP. Over the last 24 h, cells were grown in isotonic or hypotonic medium. After harvesting, luciferase activity was determined.

ferent NaCl concentrations for the last 24 h, but now in the presence or absence of dDAVP for 4 days before harvesting. Indeed, while luciferase levels were higher in samples treated with dDAVP, the relative changes in luciferase activity with changing tonicity in dDAVP-treated and -untreated samples were similar (Fig. 4). These data show that in mpkCCD cells, the effect of tonicity on AQP2 expression is independent of vasopressin receptor stimulation.

TonE in Tonicity-induced AQP2 Transcription—Regulation of AQP2 expression by tonicity through the TonE element, located 489 bp upstream of the AQP2 transcription start site, is controversial. To test the relevance of TonE in tonicity-affected AQP2 expression under our conditions, we generated pooled colonies of mpkCCD-AQP2–3.0-luc cells in which the TonE element was inactivated as reported (13), and subjected these and the mpkCCD-AQP2–3.0-luc cells to hypo- and hypertonicity. Surprisingly, changing to hypotonic (275 mOsm) or hypertonic (435 mOsm) medium revealed a similar change in luciferase activity in the TonE mutants cells as found for mpkCCD-AQP2–3.0-luc cells (Fig. 5*A*).

To further investigate this, we generated mpkCCD cells stably transfected with a construct in which luciferase expression is driven by 408 bp of the AQP2 promoter, thus lacking the TonE element (pGL3-AQP2– 0.4-luc). Following subjection to hypo- and hypertonicity, qualitatively similar changes in AQP2 transcription were found for the 3.0 and 0.4 kb promoter with both changes (Fig. 5*B*). Combined, these data indicate that TonE is not involved in the tonicity-induced regulation of AQP2 expression under the present conditions. In addition, our data suggest the presence of yet another tonicity-responsive element in the 408-bp segment of the AQP2 promoter.

Role of CREB in Tonicity-induced AQP2 Transcription—In this 408 bp segment is the CRE, located at position −210, which is involved in AVP-induced AQP2 transcription (2, 3). To test whether CRE also has a role in tonicity-induced regulation of AQP2, cells were stably transfected with pGL3-CRE(21)-luc, a construct in which luciferase transcription is driven by a promoter consisting of 21 tandemly placed CREs. Following growth and incubation with dDAVP as above, a reduced luciferase activity was observed with hypotonicity (Fig. 6*A*). From this, we conclude that hypotonicity does decrease CRE-regulated transcription.

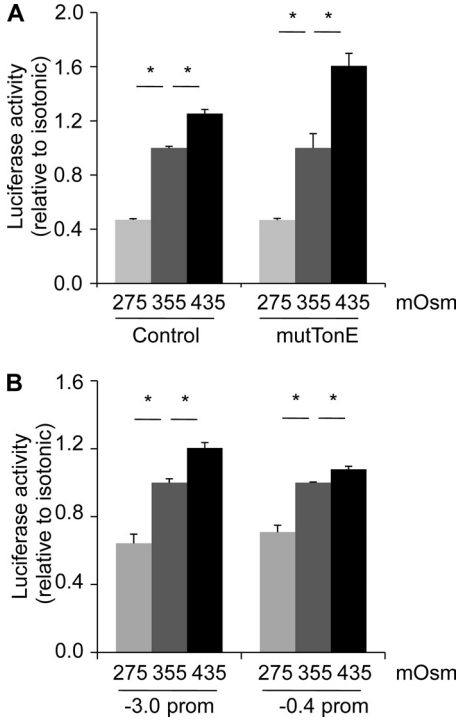

FIGURE 5. **Role of TonE in tonicity-induced AQP2 transcription.** mpkCCD cells, stably transfected with a −3.0 kb AQP2 promoter (Control) or the same promoter, containing a mutated TonE sequence (mutTonE) (*A*), or together with mpkCCD cells transfected with a −0.4 kb AQP2 promoter (*B*) were grown as above, where the last 24 h was in isotonic, hypertonic, or hypotonic medium. Cells were lysed and subjected to luciferase activity measurements. Mean values of luciferase activity (±S.E.) were determined from three independent filters per condition.

As tonicity-regulated AQP2 expression appeared to be AVP-independent (Fig. 4) while CRE-mediated transcription is changed with hypotonicity (Fig. 6*A*), we investigated which part of the AVP-cAMP-CREB-AQP2 promoter pathway was involved.

As cAMP levels show a peak right after dDAVP addition, but are reduced to low levels within 120 min following addition (28), we tested the effect of hypotonicity on dDAVP-induced cAMP levels in the long and short term. For the long term, cells were grown as above, treated with dDAVP for 4 days, incubated in hypotonic or isotonic medium during the last 24 h, and treated with the phosphodiesterase inhibitor IBMX during the last 30 min. cAMP levels were not changed by hypotonicity (Fig. 6*B*). To investigate whether there is an effect of hypotonicity on short term cAMP generation, cells were grown without dDAVP, incubated in hypotonic or isotonic medium during the last 24 h, and subsequently treated with IBMX for the last 30 min and with dDAVP for the last 15 min. Analysis revealed that the dDAVP-induced cAMP levels were not changed (Fig. 6*B*).

To test if the effect of tonicity on AQP2 transcription involves protein kinase A (PKA), we tested whether the hypotonicity-induced decrease in AQP2 transcription was still observed when we would block PKA activity with H89, a PKA-selective inhibitor. Cells were grown for 8 days and 24 h before harvesting, medium was replaced with iso- or hypotonic medium with dDAVP with or without 10 μM H89. H89 decreased luciferase activity in cells grown in both iso- and hypotonic medium, indicating that PKA is involved in AQP2

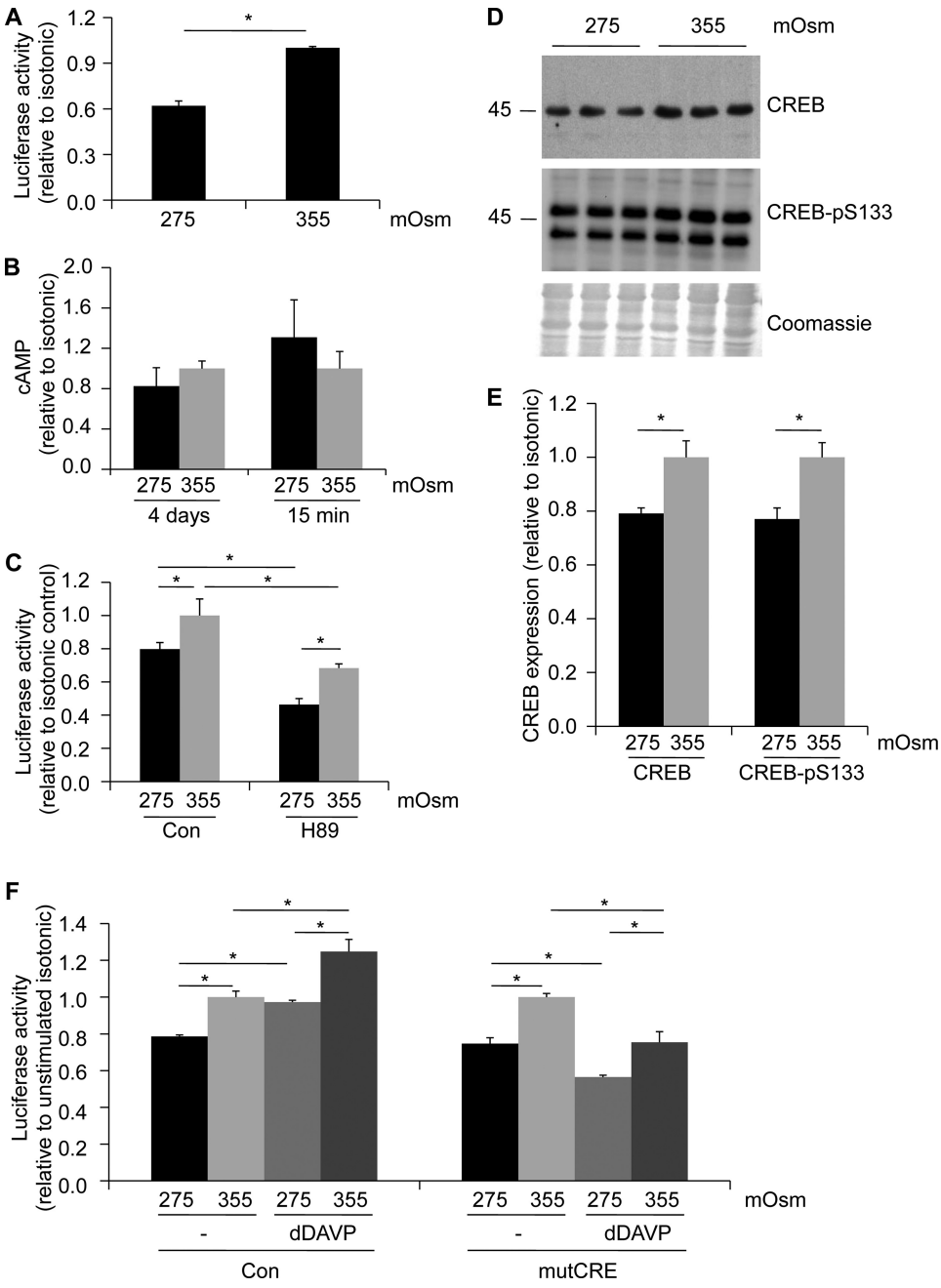

FIGURE 6. **Role of CREB in tonicity-induced AQP2 transcription.** *A*, mpkCCD cells, stably transfected with a luciferase gene driven by a multi-CRE promoter, were grown as described above and grown for the last day in hypotonic or isotonic medium in the presence of dDAVP, lysed, and analyzed for luciferase activity. *B*, mpkCCD cells were grown as above and either pretreated with dDAVP for 4 days or only treated with dDAVP 15 min before harvesting. In both conditions, cells were cultured in hypotonic or isotonic medium over the last 24 h. After harvesting, cAMP levels were measured. *C*, mpkCCD cells, stably transfected with a luciferase reporter construct containing 0.4 kb of the AQP2 promoter were grown as above. The last 24 h, cells were treated with dDAVP and the PKA inhibitor H89 (10 μM) and grown in hypotonic or isotonic medium, lysed, and analyzed for luciferase activity. *D*, mpkCCD cells were grown as described under *A*. Cells were lysed and subjected to immunoblotting for CREB and Ser-133-phosphorylated CREB (indicated). In the CREB-pS133-blot, the *upper band* represents phosphorylated CREB, whereas the *lower band* represents ATF-1, which is also recognized by this antibody. Molecular masses (in kDa) are indicated on the *left*. *E*, relative abundance of total and Ser-133-phosphorylated CREB derived from Fig. 6*D* and normalized for their expression in isotonic medium. *F*, mpkCCD cells stably transfected with constructs encoding luciferase and preceded by the −0.4 kb AQP2 promoter (*Con*) or the same promoter, containing a mutated CRE sequence (*mutCRE*), were grown as above, with or without dDAVP and the last 24 h in hypotonic or isotonic medium. After harvesting, luciferase activity was determined. Mean values (±S.E.) were determined from three independent filters per condition. Significant differences ($p < 0.05$) are indicated by an *asterisk*.

transcription at both tonicities. However, the H89-induced down-regulation of AQP2 transcription of about 30% was the same for both tonicities (Fig. 6*C*), indicating that neither PKA, nor cAMP or dDAVP are involved in the hypotonicity-induced reduction in AQP2 transcription.

A tonicity-induced decrease in transcription can be caused by a reduced CREB activity, which can be the result of a decrease in total CREB and/or CREB phosphorylation at Ser-133 (29). Indeed, immunoblotting showed a small, but significant decrease in total and CREB-pS133 with hypotonicity (Fig.

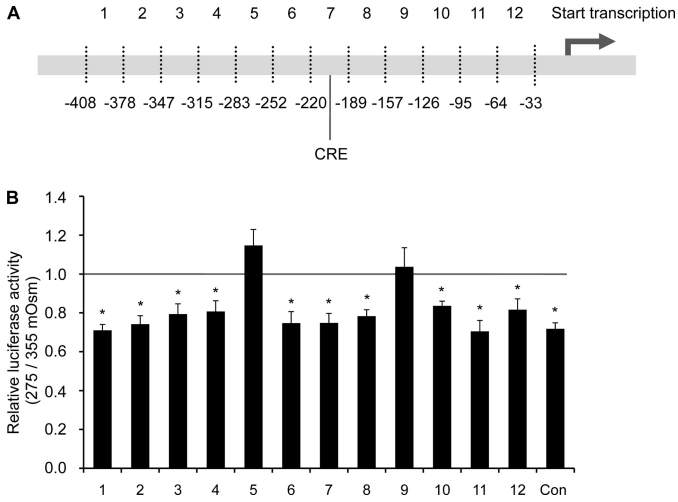

FIGURE 7. **AQP2 promoter segments involved in hypotonicity response.** *A*, position of the 31-bp segments deleted from the AQP2 promoter in relation to the transcription start site. *B*, mpkCCD cells stably transfected with a −0.4 kb AQP2 promoter construct, containing deletions as indicated in *A*, were grown as above. The last 24 h, cells were grown in hypotonic or isotonic medium. After harvesting, luciferase activity was determined. The relative luciferase activity is normalized for differences in transfection efficiencies by dividing the values obtained in the hypotonic medium by the values obtained in the isotonic medium.

6, *D* and *E*). The relative changes in total and CREB-pS133 were similar, indicating that the decrease in CREB-pS133 is due to the decrease in total CREB.

To investigate the role of the CRE element in the AQP2 promoter, mpkCCD cells were stably transfected with the pGL3-AQP2–0.4-luc construct in which the CRE element was inactivated. Subsequently, these and mpkCCD-AQP2–0.4-luc cells were subjected to the hypotonicity assay with or without dDAVP. As anticipated, dDAVP treatment increased luciferase activity significantly in mpkCCD-AQP2–0.4-luc cells, but not in cells containing the CRE mutant (Fig. 6*F*). In both cell models, however, hypotonicity led to a similar decrease in luciferase activity, which indicated that the AQP2 CRE element is not essential for the response to hypotonicity.

AQP2 Promoter Segments Involved in the Decreased Transcription under Hypotonic Conditions—Because the CRE in the AQP2 promoter was shown not to be essential for the hypotonicity effect (Fig. 6*F*), we further analyzed the −0.4kb AQP2 promoter for the hypotonicity element by generating twelve AQP2 promoter deletion constructs in which in each 31 bp (three rounds of the DNA helix) was deleted (Fig. 7*A*), leaving the relative orientation of the transcription factor binding sites intact. Cells stably transfected with these constructs were tested for their response to 24 h of hypotonicity in the presence of dDAVP. While most cells with these constructs yielded significantly reduced luciferase activity with hypotonicity, luciferase activity was not significantly reduced in cells containing constructs lacking nucleotides −284 to −254 (segment No. 5) and −158 to −127 (No. 9) (Fig. 7*B*).

DISCUSSION

mpkCCD Cells Are a Proper Model to Study Tonicity-induced Regulation of AQP2 Expression—Our data reveal that mpkCCD cells are a proper model to study hyper- and hypotonicity-induced changes in AQP2 expression and transcription. First, following transfection of a luciferase reporter construct preceded by a 3.0 kb AQP2 promoter, we showed that dDAVP induced an increase in AQP2 protein expression as well as transcription in mpkCCD cells (Fig. 1, *A* and *B*). This illustrated that the 3.0 kb promoter of AQP2 is sufficient for the response to dDAVP and is consistent with data of others using the human AQP2 promoter of a similar length in other cells (2, 3, 30, 31). Secondly, both AQP2 protein expression and transcription were increased with hypertonicity (Fig. 1, *C* and *D*), which is in line with the hypertonicity-induced increased AQP2 protein expression shown *in vivo* (9) and *in vitro* (10).

We also observed a down-regulation of AQP2 in response to hypotonicity *in vitro*. This down-regulation of AQP2 was prevented when re-adjusting osmolality with sorbitol, sucrose, or NaCl, but not urea (Fig. 1*E*). It is therefore tonicity, and not osmolality, that affects AQP2 expression, which is in agreement with previous results (10). Besides, we showed that the final tonicity and not the change in tonicity determined the extent of AQP2 transcription (Fig. 2).

Hypotonicity-induced AQP2 Down-regulation Is Independent of dDAVP, Prostaglandins, and Nitric Oxide Production—Similar to *in vivo* and other *in vitro* studies with hypertonicity (7, 9), we show that the hypotonicity-induced decrease in AQP2 expression is independent of dDAVP, as the relative effects of hypotonicity were similar in the absence or presence of dDAVP (Fig. 4).

We investigated this further. Hypotonicity did not affect the cAMP-PKA pathway, as cAMP levels were not changed by hypotonicity and PKA blockade with H89 had no effect on the hypotonicity-induced down-regulation of AQP2. The decrease in luciferase activity in cells treated with H89 shows that this drug was still active (Fig. 6*C*).

Interestingly, hypotonicity significantly decreased luciferase activity in cells possessing several CRE elements coupled to the luciferase cDNA (Fig. 6*A*), indicating that tonicity does affect CRE-mediated transcription. This decrease can be attributed to a decrease in total CREB protein levels, which was similar to the fractional decrease in CREB phosphorylation on serine 133 (Fig. 6, *D* and *E*). CRE, however, appeared not to be essential for the tonicity response of AQP2 expression, because mutating or deleting the CRE sequence in the AQP2 promoter had no effect on the tonicity-mediated AQP2 transcription (Figs. 6*F* and 7). Considering the observed reduced expression of luciferase with hypotonicity from the CRE-luciferase construct, it may be that the 21 tandemly placed CREs in this construct makes it more sensitive to detect CRE-mediated transcription than the AQP2 promoter. Alternatively, hypotonicity-regulated expression of AQP2 gene transcription is dominated by transcription factor binding sites other than its CRE. In either case, our data indicate that the CRE element in the AQP2 promoter is irrelevant for the reduced transcription of the AQP2 gene with hypotonicity, which is in line with our data that cAMP levels and PKA activity are not changed with hypotonicity (Fig. 6, *A*–*C*) and with the findings of our group and others that the effect of tonicity is AVP independent.

A candidate involved in the cellular signaling leading to the hypotonicity-mediated decrease in AQP2 transcription is NO,

as it has been shown that NO decreases AQP2 expression (32, 33) and renal NO synthase expression correlates inversely with medullary tonicity (34, 35). Similarly, Murase *et al.* (24) found that endogenous NO synthesis was significantly increased in the vasopressin-escape animals and that treatment with L-NAME decreased urine volume and partially increased AQP2 expression. In agreement with this, blocking NO synthesis by L-NAME resulted in increased AQP2 protein abundance in mpkCCD cells (Fig. 3*A*). L-NAME did not however affect the tonicity-mediated change in AQP2 abundance (Fig. 3*B*). Moreover, the tonicity-induced changes in AQP2 transcription were not changed with L-NAME (Fig. 3*C*). These data reveal that, although NO decreases AQP2 abundance in mpkCCD cells, NO does not affect AQP2 gene transcription and is not involved in the tonicity-regulated change in AQP2 expression.

Another mechanism involved might be an increased prostaglandin production. Prostaglandin E_2 is known to reduce AVP-stimulated water reabsorption in the collecting duct (25, 26). Moreover, Gross *et al.* (27) showed an increased urinary prostaglandin E_2 excretion in vasopressin-escape, and preventing this increase with indomethacin resulted in a delay in the onset of escape. In agreement with these data, blocking prostaglandin production with indomethacin resulted in an increased AQP2 protein expression in our mpkCCD cells (Fig. 3*A*). No effect was seen on AQP2 promoter-driven luciferase activity, however, indicating that the decrease of AQP2 abundance with prostaglandins occurs at the protein or mRNA stability level, rather than AQP2 gene transcription. In addition and similar to the effect of L-NAME, indomethacin treatment did not affect the tonicity-induced AQP2 expression, suggesting that prostaglandin production is not affected by tonicity, and is not involved in the tonicity-regulated expression of AQP2.

In the context of the *in vivo* data, our data indicate that paracrine-produced increased levels of prostaglandins and/or NO in the kidney may indeed contribute to the reduced AQP2 abundance observed in vasopressin-escape animals, but that they cannot explain the effect of tonicity on AQP2 gene transcription.

Tonicity-induced AQP2 Transcription Is TonE Independent—The role of TonE in tonicity-regulated AQP2 expression is controversial. In our experiments, the TonE in the AQP2 promoter was not important for AQP2 transcription regulation by tonicity, because: 1) introduction of a TonE mutation identical to the mutation introduced by Hasler *et al.* in a −3.0 kb AQP2 promoter had no effect on tonicity-induced AQP2-transcription (Fig. 5*A*) and 2) luciferase activity obtained from the −0.4 kb AQP2 promoter, which lacks TonE, and the −3.0 kb promoter were similarly influenced by the different tonicities used to culture the cells in (Fig. 5*B*).

Our data are in contrast to those obtained by Hasler *et al.* (13) who used the same cell model, but under different conditions. Growing cells for only 2 days, where the last day was in serum-free medium and without dDAVP stimulation, Hasler *et al.* found an absence of the hypertonicity-induced AQP2-promoter driven luciferase activity in mpkCCD cells when the TonE element was mutated. We tested whether this could be due to different experimental conditions. Indeed, growing cells in the same way, we saw a significant reduction, but not complete absence, in hypertonicity-induced luciferase activity in the TonE mutant cells compared with the mpkCCD-AQP2–3.0-luc cells (not shown). This difference was caused by the shorter growth period, as growing the cells for 8 days, the last 24 h with hypertonic serum-free medium without dDAVP, did not show any difference in the hypertonic response between the promoters with an intact or mutated TonE element (not shown). As in our experience, mpkCCD cells are only polarized after 4 days of culture (not shown), growing the cells for 8 days, as used in our experiments, better mimics the *in vivo* situation.

Our data seem in contrast to *in vivo* data, as TonEBP$^{-/-}$ mice and mice transgenic for dominant-negative TonEBP showed decreased AQP2 expression (15, 16). However, the severe atrophy of the renal medulla in the TonEBP$^{-/-}$ and dominant-negative TonEBP-overexpressing mice complicates the interpretation of the direct involvement of TonE in tonicity regulation of AQP2 expression. To resolve this apparent discrepancy, investigation of the role of TonE and TonEBP in a conditional knock-out mouse in which the structure of the renal medulla would be intact is needed.

Segments from −283 to −252 and −157 to −126 bp of the AQP2 Promoter Are Involved in Hypotonicity-induced AQP2 Down-regulation—Using a −0.4 kb promoter, hypo- and hypertonicity showed qualitatively similar changes in AQP2 transcription as compared with the −3.0 promoter (Fig. 5*B*), indicating that a tonicity responsive element is present in the 408 bp AQP2 promoter. With subsequent segment deletions, the location of this TonE element could be pinpointed to two 31 bp promoter segments (5 and 9) in the −0.4 kb AQP2 promoter, covering nucleotides −283 to −252 and −157 to −126 bp.

Li *et al.* (14) reported that the effect of hypertonicity on AQP2 transcription depends on NFATc sites in the AQP2 promoter. The NFATc sites tested by Li *et al.* are localized in segments 1, 6, and 7 of the −0.4 kb promoter. Two segments are located more upstream; one of them representing the TonE sequence. In our experiments, neither the upstream segments, nor segments 1, 6, or 7 appeared important in the hypotonicity response. One explanation for this difference could be the differences in experimental conditions, as Li *et al.* used unpolarized mpkCCD cells and looked at an effect of tonicity after 6 h. This time period of subjection to a changed tonicity indeed seems of relevance, because Hasler *et al.* (10) have shown that the response to a change in tonicity within 3 h is opposite to that obtained after 24 h. Another explanation could be that in the experiments performed by Li *et al.* all the NFATc sites were mutated simultaneously, while we only deleted one or two sites at once. The inactivation of multiple NFATc sites could influence the tonicity effect, whereas deletion of only one or two sites may be too small a change to detect.

To possibly identify the transcription factors involved, segments 5 and 9 were analyzed for potential transcription factor binding sites using TRANSFAC databases (36). In segment 5, binding sites for nuclear factor κB (NF-κB) and SP1 were identified, and in segment 9, a GATA binding site was found (Fig. 8).

Based on the literature, these transcription factors could mediate the change in AQP2 gene transcription with tonicity, as a study in intestinal epithelial cells suggest activation of

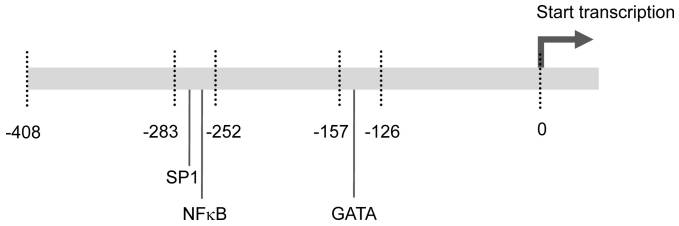

FIGURE 8. **Transcription factor binding sites.** Transcription factor binding sites identified in the −283 to −252 and −157 to −126 bp regions of the AQP2 promoter using TRANSFAC databases.

NF-κB and stimulation of NF-κB-mediated transcription by osmotic stress (37). Similarly, hypertonicity activates NF-κB in renal medullary interstitial cells, and water deprivation increases renal NF-κB-driven reporter gene expression in transgenic mice (38). In addition, GATA may play a role in tonicity-regulated AQP2 transcription, because hypertonic stress induces transcription of GATA-2 in placental trophoblast stem cells (39). It remains to be established whether these transcription factors and their promoter elements are responsible for the tonicity effect on AQP2 transcription.

In conclusion, we showed that in mpkCCD cells, tonicity-induced AQP2 expression is mediated by transcription factors other than TonEBP or CREB and that it occurs in an AVP-independent manner. The tonicity responsiveness seems to involve AQP2 promoter segments covering nucleotides −283 to −252 and −157 to −126 bp of the AQP2 promoter. Identification of transcription factors and signaling proteins involved in hypotonicity-regulated AQP2 expression in follow-up experiments will provide us better insights into physiological regulation of AQP2 expression and renal water reabsorption by tonicity and may lead to the discovery of targets for modulation of pathophysiological conditions of osmoregulation, such as with SIADH.

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Hypotonicity-induced Reduction of Aquaporin-2 Transcription in mpkCCD Cells Is Independent of the Tonicity Responsive Element, Vasopressin, and cAMP

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